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ORIGINAL ARTICLE

The Interrelationship of Pharmacologic Ascorbate Induced Cell Death and Ferroptosis

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Abstract

Pharmacologic ascorbate induced cell death and ferroptosis share common features such as iron dependency, production of ROS, lipid peroxidation, caspase independency and the possible involvement of autophagy. These observations lead us to hypothesize that ferroptosis may also be involved in cancer cell death due to pharmacologic ascorbate treatment. Thus cell death of HT-1080 cell line was induced by ferroptosis inducers and pharmacologic ascorbate then the mechanism of cell death was compared. The EC50 value of pharmacologic ascorbate on HT-1080 cell line was found to be 0.5 mM that is in the range of the most ascorbate sensitive cell lines. However either of the specific inhibitors of ferroptosis (ferrostatin-1 and liproxstatin-1) could not elevate the viability of pharmacologic ascorbate treated cells suggesting that ferroptosis was not involved in the pharmacologic ascorbate induced cell death. α-tocopherol that could effectively elevate the viability of erastin and RSL3 treated HT1080 cells failed to mitigate the cytotoxic effect of pharmacologic ascorbate further strengthened this assumption. Furthermore at lower concentrations (0.1–0.5 mM) ascorbate could avoid the effects of ferroptosis inducers. Our results indicate that pharmacologic ascorbate induced cytotoxicity and ferroptosis – albeit phenotypically they show similar traits – are governed by different mechanisms.

Keywords Pharmacologic ascorbate · Cell death · ROS · Lipid peroxidation · Ferroptosis

Introduction

Cancer cells compared to normal cells can be characterized by increased steady-state levels of ROS (reactive oxygen species) [1]. It can be ascribed to increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signalling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase or to the crosstalk with infiltrating immune cells [2]. The elevated generation of ROS can be accompanied by lower level of defence against H_2O_2. One to two orders of magnitude lower catalase activity could be measured in hepatocellular carcinoma, breast or pancreatic cancer cells than in extracts from rat liver tissue and primary rat hepatocytes. Similarly the tumour cell lines could be characterized by 10–15-fold lower GPX activity than rat liver tissue lysates or primary rat hepatocytes [3]. The anti-tumour effect of high dose ascorbate – due to its ROS generating property – was reported in several studies [1]. Ascorbate can donate an electron to a transition metal such as iron or copper. The reduced metal is capable of reacting with O_2 forming superoxide anion and then H_2O_2. In the presence of pharmacologic (mM) concentrations of ascorbate, H_2O_2 can readily react with further transition metal ions in the Fenton reaction to form the highly reactive, cytotoxic hydroxyl radical [1, 4]. The generated H_2O_2 and hydroxyl radical may cause DNA damages that is followed by the activation of poly(ADP-ribose) polymerase-1, the depletion of NAD^+ and ATP [1, 5]. Since the concentration of ascorbate in the human body is controlled (and limited) via its strictly regulated transport, pharmacologic ascorbate concentration can only be reached by its parenteral administration [1, 6]. The ascorbate induced cell death of cancer cells

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depends on H_2O_2 production [7–9] mediated by extracellular ascorbate oxidation since it can be avoided by exogenous catalase or adenoviral-mediated overexpression of catalase or GPX1 [10].

Albeit the fact that ascorbate induces cancer cell death is well known and documented thoroughly, its exact mechanism has not been unravelled yet. Several studies reported that pharmacologic ascorbate induced cancer cell death is a caspase independent apoptosis [1, 11–13]. Later, on the base of increased LC3-II level and formation of autophagosomes due to pharmacologic ascorbate treatment autophagy has also been proposed as a potential high-dose ascorbate-induced cell death mechanism [14–16].

A group of chemical compounds such as erastin and RSL3 could induce a novel form of programmed cell death of RAS-mutant tumour cells [17, 18]. The cell death induced by these compounds could be inhibited by an iron chelator (deferoxamine) and by an antioxidant, vitamin E, confirming that this form of cell death is related to intracellular iron and ROS [18]. Because of its iron-dependency it was latter called "ferroptosis" [19]. The morphology, biochemistry and genetics of ferroptosis differs considerably from all other known cell death types, such as apoptosis, necrosis, and autophagy [18, 19]. Recently it has been suggested that autophagy is involved in lipid ROS-mediated ferroptotic cell death [20]. It was reported that a number of tumour cells, such as diffuse large B cell lymphoma, renal cell carcinoma, liver cancer, cervical carcinoma, osteosarcoma and prostate adenocarcinoma cells [21, 22] are very susceptible to this iron dependent ROS involved cell death. Shortly after the description of ferroptosis it was reported that sorafenib, an inhibitor of oncogenic kinases induced ferroptosis in hepatocellular carcinoma cells [23]; then this observation was extended to other cancer cell lines [24]. Interestingly ascorbate could act synergistically with sorafenib and gives the possibility to broaden its therapeutic range [3].

The observations that ferroptosis is an iron-dependent ROS mediated cell death mechanism that could be suppressed by co-treatment with the iron chelator deferoxamine [19], and that it was not consistently modulated by inhibitors of caspase, cathepsin, or calpain proteases (z-VAD-fmk, E64d, or ALLN) [19], and autophagy is involved in its induction through the elevation of labile iron pool [20] lead us to hypothesize that ferroptosis (at least partly) is responsible for the high ascorbate dose-induced cytotoxicity in cancer cells.

Materials and Methods

Materials

100 mM ascorbic acid (ASC, Sigma®) and 100 mM reduced glutathione (GSH, Sigma®) stock solutions were prepared in complete culture medium and the pH was adjusted to phenol red neutral with sodium hydroxide. Desferoxamine mesylate (DFO, Sigma®) was dissolved in complete culture medium. Sodium pyruvate (Pyr, Sigma®) was dissolved in PBS. Ferrostatin-1 (Selleckchem), liproxstatin-1 (Selleckchem), necrostatin-1 (Selleckchem), Z-VAD-FMK (Selleckchem), erastin (Selleckchem) and RSL3 (Selleckchem) stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma®). DL-α-tocopherol acetate (Sigma®) stock solution was prepared in ethanol. Ferric 8-hydroxyquinoline (Fe(HQ)2) was prepared by combining equal volumes of ferric chloride (10 mM, Sigma®) and 8-hydroxyquinoline (20 mM, Sigma®) dissolved in DMSO.

Cell Culture

Cells used in the experiments (HT-1080 and MCF-7) were cultured according to ATCC guidelines. The complete culture medium comprised of high glucose DMEM (Sigma® AQmedia™) supplemented with 10% fetal bovine serum (Sigma®), 1% non-essential amino acids (Sigma®) and 50 μg/ml gentamicin (Sigma®). Cells were subcultured routinely before reaching 100% confluence usually in a ratio of 1:6. Cell viability was determined by MTT assay as described earlier [25].

Measurement of Viable Cell Number, Viability and Labile Iron Pool Using Flow Cytometry

Cells were seeded at a density of 0.55×10^6 cells / petri on a 6 cm tissue culture treated petri dish (Thermo Scientific™ BioLite) in complete culture medium. After 24 h of incubation the complete culture medium was renewed and supplemented with various compounds for treatment. After the desired treatment time interval, the culture medium was discarded, the cells were washed twice with PBS, trypsinised and resuspended in culture medium. A suitable volume from the cell suspension was used for the determination of viable cell number and viability using the Muse® Count & Viability Assay Kit according to the manufacturers' guidelines using a Muse® Cell Analyser.

For labile iron pool (LIP) measurements another volume of the cell suspension (with approximately 0.2×10^6 cells) was centrifuged and the cells were resuspended in HBSS with 1 μM Phen Green SK (PG-SK, Thermo Scientific™) (as used by Du et al. 2015) and incubated at 37 °C for 15 min. For positive control samples 10 μM ferric 8-hydroxyquinoline ($Fe(HQ)_2$) was further added with PG-SK. After incubation samples were subjected to flow cytometry using a Muse® Cell Analyser with the "Oxidative Stress protocol" as it uses a suitable emission filter (576 nm). Data was analysed using FlowJo®, cell population was gated using unlabelled cells.

Measurement of ROS, Lipid Peroxidation and Cell Membrane Integrity Using Flow Cytometry

Cells were seeded at a density of 0.25×10^6 cells / well on a 6 well tissue culture treated plate (Thermo Scientific™ BioLite) in complete culture medium. After 24 h of incubation the complete culture medium was discarded, the cells were washed twice with PBS and incubated in HBSS with either 10 μM dichlorofluorescein-diacetate (DCF, Thermo Scientific™) or 2 μM BODIPY™ 581/591 C11 (Bodipy-C11, Thermo Scientific™) (as used by Dixon et al. 2012) for 30 min in the cell culture incubator. After loading the dyes the cells were treated with various compounds in complete culture medium. After the desired treatment time interval, the culture medium was discarded, the cells were washed twice with PBS, trypsinised and resuspended in culture medium. Cells were then centrifuged at 300 *g*, resuspended in HBSS and transferred to FACS tubes on ice. For propidium iodide (PI, Sigma®) staining the cells were stained with 1 μg/ml PI. Cells were analysed in a BD FACSCalibur™ flow cytometer. The cell population was focused with unstained cells and all dyes were used separately to avoid interference. A minimum of 10,000 cells were analysed per condition. Data was analysed using FlowJo®, cell population was gated using unlabelled cells.

Isolation and Quantitation of Protein Samples

Cells were treated and prepared as described above and were lysed in RIPA protein isolation buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 8,0) supplemented with 1% protease inhibitor cocktail (Sigma®), 1% phosphatase inhibitor cocktail (Sigma®) and 1 mM PMSF. Samples were incubated on ice for 30 min and centrifuged at 14'000 g for 15 min at 4 °C. The supernatant was used for protein analysis and stored at −80 °C. Protein samples were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™) according to the manufacturers guidelines.

Western Blot

SDS-PAGE was done by using Hoefer miniVE (Amersham). Proteins were transferred onto Millipore 0.45 μM PVDF membrane. Immunoblotting was performed using TBS Tween (0.1%), containing 5% non-fat dry milk for blocking membrane and for antibody solutions. Loading was controlled by developing membranes for GAPDH in each experiment. For each experiment at least three independent measurements were carried out. The following antibodies were applied: antiLC3 (Proteintech, 12135-1-AP), antiPARP (Cell Signaling, 9542S), antiRIPK1 (Proteintech, 17519-1-AP) and antiGAPDH (Santa Cruz, 6C5), HRP conjugated secondary antibodies (Cell Signaling, 7074S, 7076S). The bands were visualised using chemiluminescence detection kit (Thermo Scientific, 32,106). For densitometry analysis Western blot data were acquired using ImageJ software.

Measurement of H_2O_2 Production and Oxygen Levels with an Oxygraph

These experiments were carried out in cell free conditions following the method of Du et al. 2010. Complete culture medium supplemented with various compounds was added to 6 cm petri dishes and incubated in a cell culture incubator. A suitable volume of the culture medium was sampled after a desired time interval for H_2O_2 and O_2 determination. The culture medium sample was added to the chamber of a calibrated Clark-type oxygraph (Hansatech), sealed with a plug and the initial O_2 level of the sample was recorded. 1 k U/ml catalase (Sigma®, dissolved in complete culture medium) was added to the sealed chamber using a Hamilton® pipette and O_2 evolution was monitored. The amount of the evolved O_2 is proportional to the H_2O_2 present in the sample and it was quantified utilising known H_2O_2 concentrations.

Brightfield Microscopy

Cells were seeded at 20 k cell /well density on 8 well Nunc™ Lab-Tek™ II Chamber Slide™ System (Thermo Scientific™) in complete culture medium. After 24 h cells were treated as indicated and observed using a Nikon TS2R-FL inverted microscope using a 20x phase contrast objective and a DS-Ri2 camera.

Statistical Analyses

All statistical analyses were carried out using Microsoft Excel. *P* values were calculated with unpaired Student's *t* test. Data are presented as mean ± SD from 3 independent experiments.

Results

EC50 Value and Time Course of the Cytotoxicity of Pharmacologic Ascorbate on HT-1080 Cells

First, the sensitivity of the N-RAS-mutant HT-1080 fibrosarcoma cell line towards ascorbate was determined to investigate the possible involvement of ferroptosis in pharmacologic ascorbate induced cell death. The EC50 value of ascorbate was around 0.5 mM. Ascorbate concentrations above 1.0 mM decreased the viability of cells to practically zero after 24 h of treatment (Fig. 1). Ascorbate concentrations above 0.5 mM had a significant

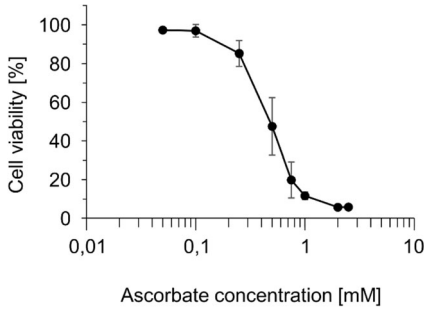

Fig. 1 The effect of ascorbate on the cell viability of HT-1080 fibrosarcoma cell line. Cells were seeded in a 96 well plate, treated with various concentrations of ascorbate for 24 h and viability was measured afterwards by the MTT cell viability assay. Absorbance values were normalised to untreated controls. The data show normalised means to untreated wells (Δ) ± SD of three independent experiments

cytotoxic effect on HT-1080 cells (Fig. 1) which could already be shown after 3 h of treatment (Fig. 2a). Dying cells appeared between 2 and 3 h of treatment with minimal dead cell count at 2 h (Fig. 2b).

Relationship of Ferroptosis and Pharmacologic Ascorbate Induced Cell Death

In the second turn of our experiments the effect of specific ferroptosis inhibitors (liproxstatin-1 and ferrostatin-1) was examined on the viability of N-RAS-mutant HT-1080 and non-RAS-mutant MCF-7 cell lines treated by a lethal dose (1.2 mM) of pharmacologic ascorbate or different ferroptosis inducers (RSL3 and erastin). The MCF-7 cell line was chosen to verify the RAS-oncogene-selectivity of ferroptosis inducers.

The viability of both cell lines decreased significantly by high dose ascorbate (1.2 mM) (Fig. 3). However neither ferrostatin-1 nor liproxstatin-1, the specific inhibitors of ferroptosis could elevate the viability of either cell lines (Fig. 3). The effectiveness of these inhibitors was tested for lower doses of ascorbate, where they also lacked effectivity (data not shown). Glutathione (GSH) was the sole effective inhibitor of 1.2 mM ascorbate induced cell death in both cell lines and the iron chelator deferoxamine (DFO) could moderately mitigate

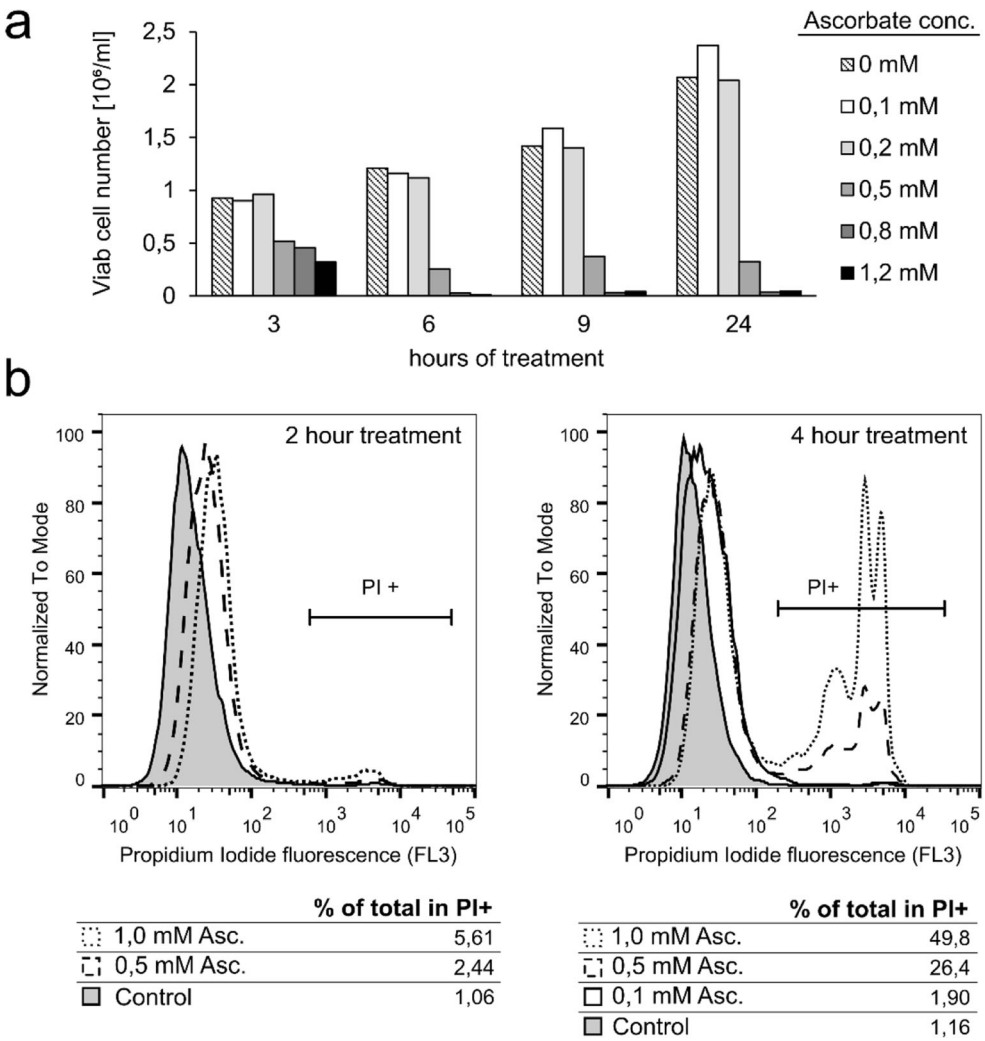

Fig. 2 Time course of ascorbate induced cytotoxicity on HT-1080 cells. Cells were seeded in 6 well plates and treated with various concentrations of ascorbate for the desired time. After treatment cells were detached with trypsin and viable cell numbers were determined by flow cytometry **a** Muse® Cell Analyzer with a suitable Count & Viability kit according to the manufacturer's guidelines or **b** FACSCalibur® with 1 μg/ml propidium iodide with left panel showing cytotoxicity after 2 h and right panel cytotoxicity after 4 h). Control (Ctr.) samples were fluorescent labelled but untreated

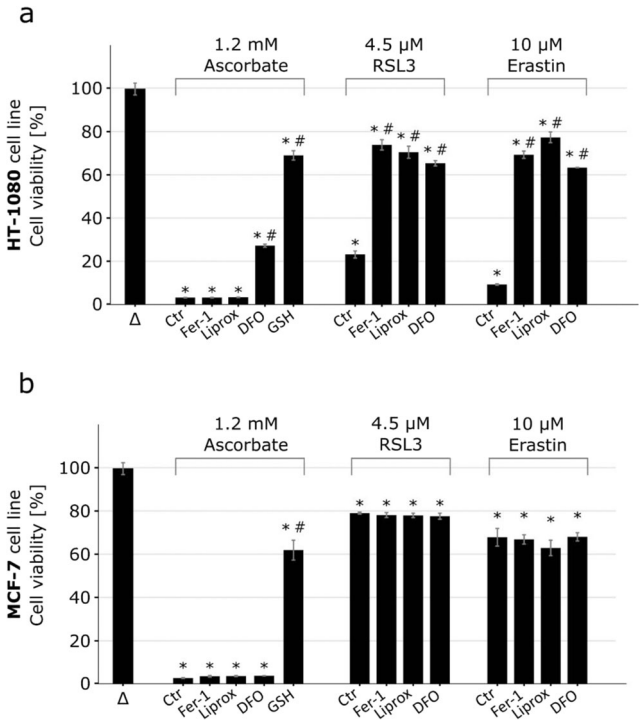

Fig. 3 Cell viability of **a** the RAS-mutant HT-1080 fibrosarcoma and **b** the non-RAS-mutant MCF-7 adenocarcinoma cell lines in response to ascorbate and ferroptosis inducer treatment measured by the MTT cell viability assay. Cells were treated as indicated with the following concentrations: ascorbate 1.2 mM, ferrostatin-1 10 μM (Fer-1), liproxstatin-1200 nM (Lipprox), deferoxamine 250 μM (DFO), glutathione 5 mM (GSH), RSL3 4.5 μM, erastin 10 μM; The data show normalised means to untreated wells (Δ) ± SD of three independent experiments; *significantly different ($P < 0,05$) from untreated (Δ); #significantly different ($P < 0,05$) from group control (Ctr. of either 1.2 mM Ascorbate or 1 μg/ml RSL3 or 10 μM Erastin)

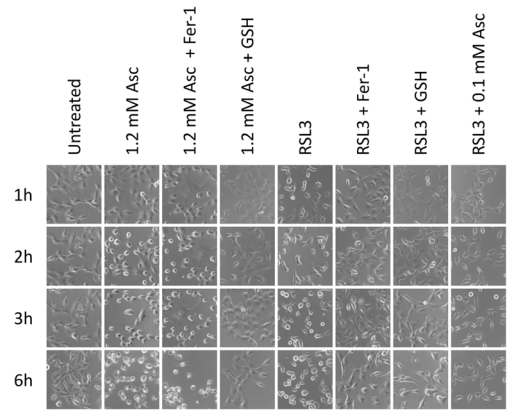

Fig. 4 Time course investigation of the change in cell morphology induced by high dose ascorbate (1.2 mM Asc) or RSL3 (1 μg/ml) with or without the inhibitors ferrostatin-1 (Fer-1, 5 μM), glutathione (GSH, 1 mM) or low dose ascorbate (0.1 mM Asc). Both high dose ascorbate and RSL3 induce cell rounding which is most notable at 3 h after treatment. Ferrostatin-1 can inhibit the effect of RSL3, but not that of high dose ascorbate, while glutathione is effective against both compounds. Low dose ascorbate was also able to protect cells from RSL3 induced change in morphology which support its inhibitory effect

it but only in HT-1080 cells (Fig. 3). GSH and DFO was previously shown to inhibit ferroptosis induced by RSL3 and erastin which partly led us to hypothesise an interrelationship between the two cell deaths. However, the anti-ferroptotic lipid-peroxide scavenger α-tocopherol (50 nM) did not show any inhibitory effect (data not shown).

Both cell lines were also treated with the known ferroptosis inducers, RSL3 and erastin (Fig. 3a-b) as a positive control for ferroptosis. As expected, ferroptosis could be induced in the RAS-mutant HT-1080 cells by both inducers that could be inhibited by specific inhibitors ferrostatin-1 and liproxstatin-1, and the iron chelator DFO was also effective (Fig. 3a). Ferroptosis inducers somewhat decreased the cell viability of MCF-7 cells, but ferroptosis inhibitors did not counteract this effect (Fig. 3b). This suggested that the non-RAS-mutant MCF-7 cell line was insensitive towards the ferroptosis inducer compounds possibly as a consequence of its' genotype.

The investigation of cell morphology showed that the shape of cells became rounded due to pharmacological ascorbate or RSL3 treatment even 3 h after the treatment (Fig. 4). This change in cell morphology could only be prevented by GSH in the case of pharmacologic ascorbate treatment. The addition of specific ferroptosis inhibitor ferrostatin-1 could not alleviate the effect of pharmacologic ascorbate (Fig. 4). In the case of RSL3 treatment this change in cell morphology could be prevented by the specific ferroptois inhibitor ferrostain-1, GSH or by low dose ascorbate (0.1 mM) (Fig. 4). All these morphological observations are in accordance with the results of cell viability assays (Figs. 2 and 3).

To investigate the mechanism of the inhibitory effect of GSH and DFO on high dose ascorbate induced cytotoxicity the time course of H_2O_2 production was monitored in a cell free culture medium. H_2O_2 was produced in the culture medium as a consequence of the presence of ascorbate (Fig. 5a). The peak of H_2O_2 production could be observed after 60 min of incubation and the presence of GSH or pyruvate diminished the measurable H_2O_2 (H_2O_2 levels below 100 μM are below the quantification limit of this method) which in term could be responsible for their inhibitory effect. The production rate of H_2O_2 (the consumption of O_2) was similar in all four samples (Fig. 5b).

The Role of Alternative Programmed Cell Death Pathways in Pharmacologic Ascorbate Induced Cytotoxicity

The ferroptosis inhibitors liproxstatin-1 and ferrostatin-1 did not show any protective effect, hence the possibility of alternative cell death pathways was investigated. First we tested the inhibitory effect of various known programmed cell death inhibitors at various concentrations against pharmacologic ascorbate induced loss in cell viability: wortmannin and bafilomycin A1 for autophagy, necrostatin-1 for necroptosis

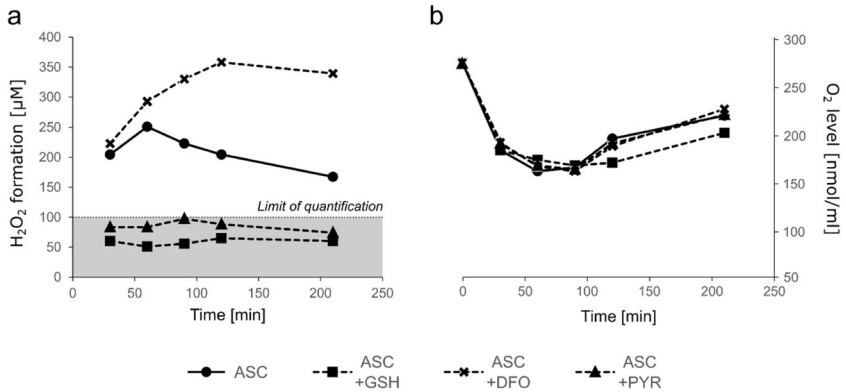

Fig. 5 **a** Time course production of H_2O_2 by ascorbate and **b** corresponding oxygen levels in the medium with or without inhibitors. Measured in a cell-free culture medium with a calibrated Clark-type oxygraph instrument. H_2O_2 level was calculated from O_2 evolution in response to catalase addition using the calibration curve: H_2O_2 (μM) = 0.215 * O_2 (nmol/ml) - 11. H_2O_2 levels above 100 μM were readily quantifiable. The following concentrations were used: ascorbate 1 mM, glutathione 5 mM, deferoxamine 250 μM, pyruvate 5 mM

and Z-VAD-FMK for apoptosis. The inhibitors were tested against a range of ascorbate concentrations from sub-lethal (0.1 mM) to lethal (above 1.0 mM) doses using the HT-1080 N-RAS-mutant fibrosarcoma cell line. We found that autophagy inhibitors wortmannin and bafilomycin A1 and necroptosis inhibitor necrostatin-1 showed a moderate inhibitory effect but only against moderate concentration of ascorbate (0.5 mM), the protective effect of all inhibitors was lost above 1.0 mM of ascorbate (Fig. 6.). These results confirmed previous findings which showed the implication of the autophagic and necroptotic pathway in pharmacologic ascorbate induced cell death.

In order to further investigate the involvement of these pathways we examined the activation of protein markers of key cell death mechanisms (i.e. autophagy, apoptosis and necroptosis) upon ascorbate treatment (Fig. 7.). Protein samples were analysed after 2 h of treatment with ascorbate which was previously determined as the onset of toxicity (Fig. 2b). The ratio of LC3 II/LC3I levels was measured to assess autophagy. Both 0.1 and 0.6 mM ascorbate increased the ratio of LC3II/LC3I (Fig. 7.). To confirm autophagy induction cells were pre-treated with bafilomycin A1 which was able to further increase LC3 II/LC3I in a combined treatment (i.e. addition of both ascorbate and bafilomycin A1) (Fig. 7.) supposing the presence of autophagic response during ascorbate treatment. Necroptosis marker, RIPK1 was also shown a significant increase; however its induction was supressed when cells were pre-treated with bafilomycin A1 (Fig. 7.). To detect apoptosis PARP cleavage was determined for caspase activation. Significant PARP cleavage was not detected suggesting that apoptosis was not involved in neither ascorbate treatment nor ferroptosis induction (Fig. 7.). These results further confirmed that both autophagy and necroptosis, but not apoptosis are involved in pharmacological ascorbate induced cell death.

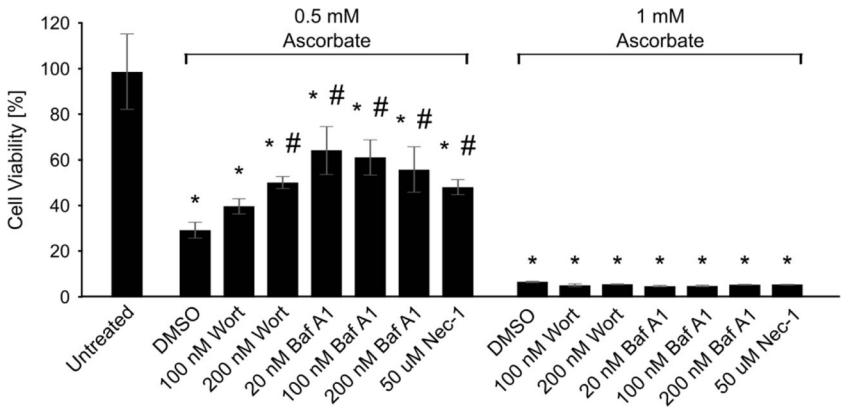

Fig. 6 Cell viability of HT-1080 fibrosarcoma cell lines in response to low dose (0.5 mM) and high dose (1.0 mM) ascorbate measured by the MTT cell viability assay. Cells were treated as indicated with the following concentrations: ascorbate 0.5 and 1.0 mM, wortmannin (Wort) 100 nM and 200 nM, Bafilomycin A1 (Baf A1) 20 nM, 100 nm and 200 nM, necrostatin-1 (Nec-1) 50 μM; The data show normalised means to untreated wells (Δ) ± SD of three independent experiments; *significantly different ($P < 0,05$) from untreated (Δ); #significantly different ($P < 0,05$) from group control (Ctr. of either 0.5 or 1.0 mM Ascorbate)

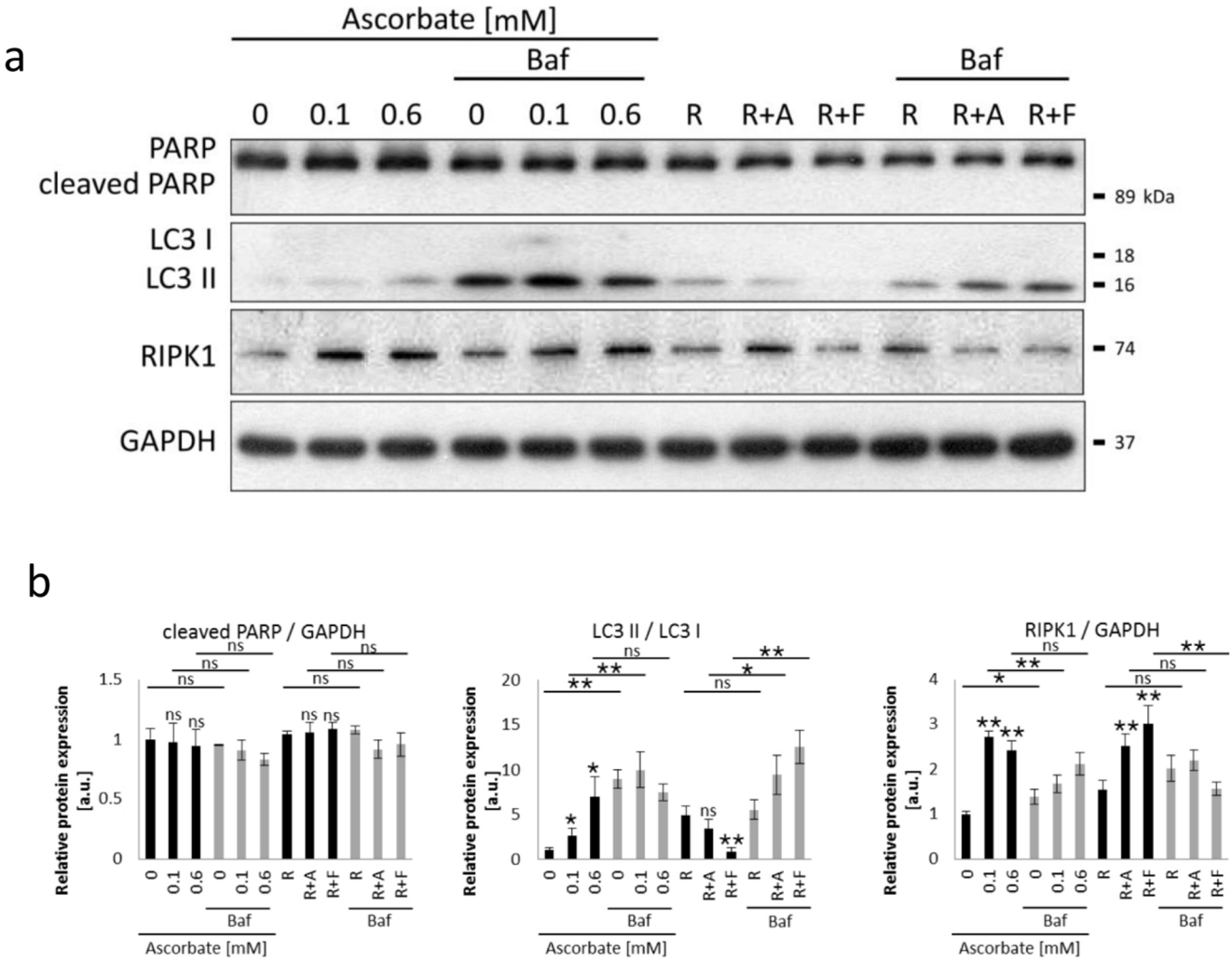

Fig. 7 Detecting the key cell death markers on protein level. **a** Westernblot analysis of apoptosis (via PARP cleavage), autophagy (via appearance of LC3II) and necroptosis (via appearance of RIPK1). HT-1080 fibrosarcoma cells were treated as indicated with the following concentrations: ascorbate 0.1 and 0.6 mM (A), 20 nM, Bafilomycin A1 (Baf); and 4.5 μM RSL3 (R) combined with 0.6 mM ascorbate or ferrostatin-1 (F) 10 μM **b** Densitometry data represent the intensity of cleaved PARP and RIPK1 normalised for GAPDH and LC3II normalized for LC3I. For each of the experiments, three independent measurements were carried out. Error bars represent standard deviation, asterisks indicate statistically significant difference from the control: ⋆ - $p < 0.05$; ⋆⋆ - $p < 0.01$

Ascorbate Does Not Act Synergistically with Ferroptosis Inducers, but it is an Inhibitor of Ferroptosis

Since pharmacologic ascorbate induced cytotoxicity and ferroptosis are both similarly characterised as an iron-dependent oxidative cell death, we hypothesised whether there is any interference between the two mechanisms. Therefore, the effect of co-treatment of the RAS-mutant HT-1080 cell line with ascorbate and the ferroptosis activators RSL3 and erastin was investigated. At lower concentrations (0.1–0.5 mM) ascorbate behaved as an inhibitor of both RSL3 and erastin induced ferroptosis (Fig. 8). RSL3 induced ferroptosis was significantly inhibited by ascorbate concentrations at as low as 0.1 mM of ascorbate (Fig. 8a) while the inhibition of erastin induced ferroptosis required higher concentration of ascorbate (0.5 mM) (Fig. 8a). At higher ascorbate concentrations, the cytotoxic effect of ascorbate exceeded its protective effect (Fig. 8a). This cytotoxic effect was countered when pyruvate was also present in the medium consequently ascorbate concentration could be further increased in order to further protect cells from ferroptosis (Fig. 8a-b). Pyruvate alone had no effect on the viability of cells when co-treated with RSL3 or erastin (data not shown).

Features of Ferroptosis Inhibition and Ascorbate Induced Cytotoxicity

Our results show that both pharmacologic ascorbate and RSL3 induce ROS and lipid peroxide formation (Fig. 9). The co-treatment of RSL3 treated cells with low dose (0.1 mM) ascorbate significantly decreased the level of both

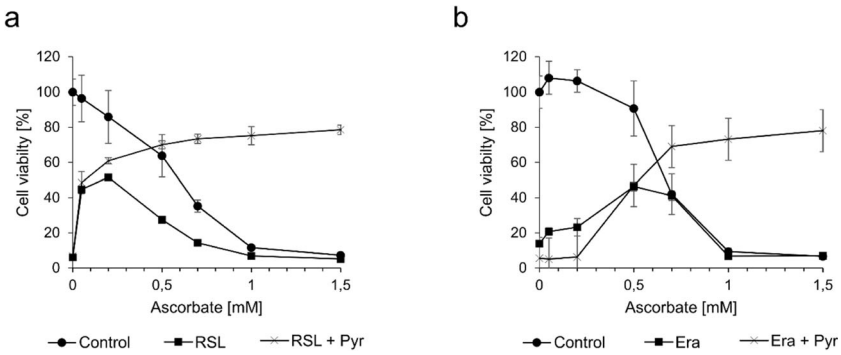

Fig. 8 Cell viability of HT-1080 cells treated with various concentrations of ascorbate, 5 mM pyruvate and co-treated with either **a** 0.5 μg/ml RSL3 or **b** 5 μM erastin. The data show normalised means to untreated wells (Δ) ± SD of three independent experiments

ROS and lipid peroxide formation (Fig. 9). The production of ROS (Fig. 9a) LOOX (Fig. 9b) and the elevation of labile iron pool (LIP) (Fig. 9c) due to pharmacologic ascorbate treatment was concentration dependent. Ascorbate concentration as low as 0.25 mM induced detectable levels of ROS while cells treated with 0.1 mM ascorbate showed ROS levels similar to untreated cells (Fig. 9d).

Discussion

The observations that pharmacologic ascorbate and erastin, RSL3 induced cell deaths share common features such as iron dependency, production of ROS, lipid peroxidation, GSH depletion [26], caspase independency [19] and the possible involvement of autophagy [1] [20] lead us to hypothesize that cancer cell death due to high concentration ascorbate treatment also has ferroptotic component. The main object of our study was the HT-1080 cell line since ferroptosis was originally described on this cell line [19]. The EC50 value of pharmacologic ascorbate on HT-1080 cell line was first described by our group. It was found to be 0.5 mM (Fig. 1) that was in the range of the most sensitive cell lines to high dose ascorbate treatment, such as human lymphoma (JLP-119), glioblastoma (S635) cell lines, murine neuroblastoma (2a), breast cancer (TS1A), (4 T1) cell lines and rat glioblastoma (RG2), (9 L), (C6) cell lines [7, 8]. Dying cells appeared between 2 and 3 h of treatment with minimal dead cell count at 2 h (Fig. 2b). Similar to the JLP-119 human lymphoma line [7] ascorbate concentrations as low as 0.1 mM did not show any cytotoxic effect on HT-1080 cells (Fig. 2b).

In the second turn of our experiments the effect of specific inhibitors of different cell death mechanisms was examined on the viability of RAS-mutant HT-1080 and non-RAS-mutant

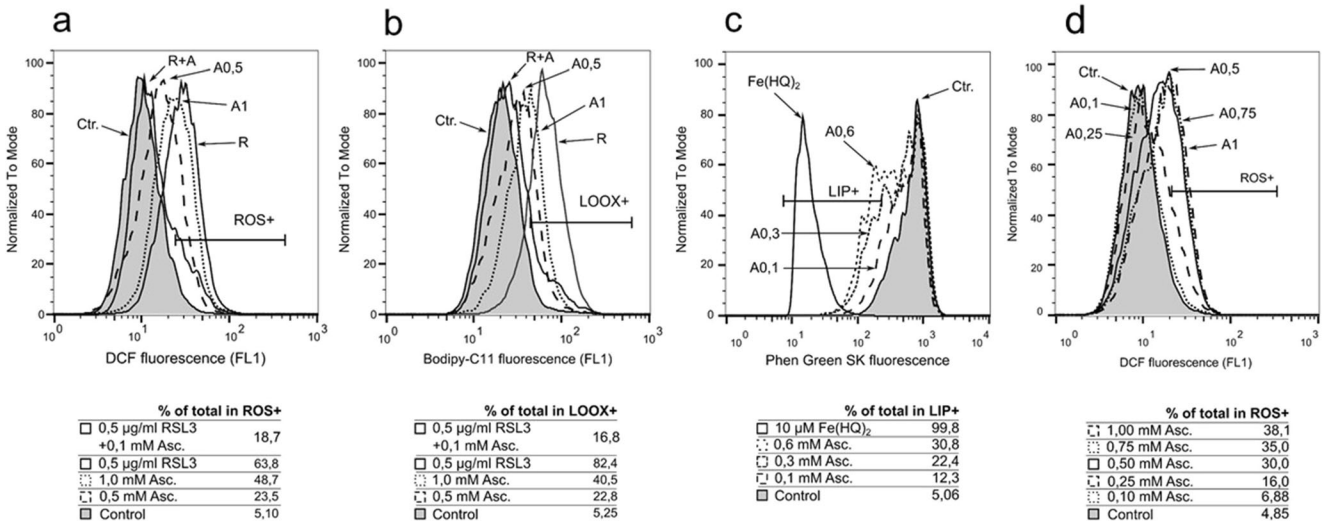

Fig. 9 Flow cytometric analysis of the effect of ascorbate and RSL3 after 2 h of treatment. Both ascorbate - at concentrations of 0.5 (A0.5) and 1 mM (A1) - and 0.5 μg/ml RSL3 (R) cause oxidative stress through ROS formation (**a**) and lipid peroxidation (**b**). The effect of 0.5 μg/ml RSL3 is countered by co-treatment with 0,1 mM ascorbate (R + A) (**a**-**b**). Ascorbate also induces the elevation of the labile iron pool (LIP) in a dose dependent manner (0.1 mM = A0.1, 0.3 mM = A0.3, 0.6 mM = A0.6) (**c**). 10 μM ferric 8-hydroxyquinoline ($Fe(HQ)_2$) was used as a positive control. Control (Ctr.) samples were fluorescent labelled but untreated. **d** ascorbate induced ROS formation after 4 h of treatment. Cells were treated with various concentrations of ascorbate (0.1–1 mM, denoted by the letter "A" followed by the concentration of ascorbate in mM). Control (Ctr.) samples were fluorescent labelled but untreated

MCF-7 cell lines treated by pharmacologic ascorbate and different ferroptosis inducers. The significantly decreased viability of both cell lines due to pharmacologic ascorbate treatment (Fig. 3) could not be avoided or mitigated by the specific inhibitors of ferroptosis (ferrostatin-1 and liproxstatin-1) [19, 27] (Fig. 3) suggesting that ferroptosis was not involved in the pharmacologic ascorbate induced cell death. Similar observations could be taken by the investigation of the morphology of HT-1080 cells due to pharmacologic ascorbate or RSL3 treatment (Fig. 4). This assumption was further strengthened by the ineffectiveness of the lipophilic antioxidant α-tocopherol, that could effectively elevate the viability of erastin and RSL3 treated HT1080 cells [19, 23]. GSH was the sole effective inhibitor of ascorbate induced cell death in both cell lines and deferoxamine (DFO) could moderately mitigate it but only in HT-1080 cells (Fig. 3). The inhibitory effect of DFO in HT-1080 cells suggests a cell line specific protective mechanism.

According to the earlier observations of Dixon et al. [19] ferroptosis could be induced in the RAS-mutant HT-1080 cells by both known ferroptosis inducers (RSL3 and erastin) and it could be inhibited by the known specific inhibitors ferrostatin-1 and liproxstatin-1, while the iron chelator DFO was also effective (Fig. 3a). Although ferroptosis inducers somewhat decreased the cell viability of the non-RAS-mutant MCF-7 cell line, ferroptosis inhibitors did not counteract this effect hence it can be considered non-specific (Fig. 3b). Albeit a study found ferroptosis in MCF-7 cells after siramesine and lapatinib treatment, unfortunately erastin as a standard ferroptosis inducer treatment was not performed [28]. It cannot be ruled out that ferroptosis can be induced by different mechanisms with the different inducers.

Summarily the specific ferroptosis inhibitors ferrostatin-1 and liproxstatin-1 had no significant effect on ascorbate induced cytotoxicity in either cell line. These results indicate that pharmacologic ascorbate induced cytotoxicity and ferroptosis – albeit phenotypically they show similar traits – are governed by different mechanisms.

Ascorbate reacts with molecular oxygen in the presence of ferrous iron (Fe^{2+}) to form superoxide anion ($O_2^{\cdot-}$) which in term forms hydrogen peroxide (H_2O_2) [8, 9]. This reaction can take place in the culture medium of the cells and the formed H_2O_2 is considered to be responsible for the oxidative damage occurring with ascorbate induced cytotoxicity as catalase supplementation alleviates cell death [7, 10]. In order to investigate the mechanism of the inhibitory effect of GSH and DFO on high dose ascorbate induced cytotoxicity the time course of H_2O_2 production was monitored in a cell free culture medium. Our results corroborate the findings [7–9] that H_2O_2 is produced in the culture medium as a consequence of the presence of ascorbate (Fig. 5a). The production of H_2O_2 reached its peak at 60 min of incubation and the presence of GSH or pyruvate diminished the measurable H_2O_2 which in term could be responsible for their inhibitory effect. The effect of both compounds was expected since GSH can donate electrons to H_2O_2 even in a pure chemical reaction in the absence of any enzyme [29]. Pyruvate eliminates H_2O_2 via a decarboxylation, hence it behaves as an excellent H_2O_2 scavenger [30]. The addition of DFO resulted in an enhanced level of H_2O_2 that was sustained for the whole investigation. DFO chelates only the ferri (Fe III) form, but not the ferro (Fe II) form of iron. Therefore, the tight binding of Fe (III) by DFO prevents redox cycling, hence the conversion of H_2O_2 to hydroxyl radial (Fenton reaction) that can result in higher suspended H_2O_2 level [31]. This is counter intuitive to its cytoprotective effect in the HT-1080 cell line and suggests a more complex inhibitory mechanism. Oxygen levels measured from the culture medium from all samples show similar pattern (Fig. 5b) that reinforce the discussion above. The production rate of H_2O_2 (the consumption of O_2) is similar in all four samples (Fig. 5b) however GSH decreases the steady state level of H_2O_2 by reduction and pyruvate decreases it by the decarboxylation reaction (Fig. 5a). DFO ceases the redox cycling of Fe (II) – Fe (III) hence inhibits the consumption of H_2O_2 (the formation of hydroxyl radical), thus it elevates the steady state level of H_2O_2 (Fig. 5a).

To clarify the detailed anti-tumour mechanism of pharmacologic ascorbate, the inhibitory effect of various known programmed cell death inhibitors were tested. Autophagy inhibitors, wortmannin and bafilomycin A1 [20] and necroptosis inhibitor necrostatin-1 [32] showed a moderate inhibitory effect, but only at moderate concentration of ascorbate (0.5 mM) treatment and the protective effect of all inhibitors was lost above 1.0 mM of ascorbate (Fig. 6.). These results confirmed previous findings that autophagic and necroptotic pathways are involved in pharmacologic ascorbate induced cell death [1]. These experiments were supplemented with the investigation of protein markers of key cell death mechanisms (i.e. autophagy, apoptosis and necroptosis) upon ascorbate treatment (Fig. 7.). Ascorbate increased the ratio of LC3II/LC3I supposing the presence of autophagic response due to ascorbate treatment. The pre-treatment of the samples with bafilomycin A1 resulted in the elevation of LC3II/LC3I that further strengthened the induction of autophagy (Fig. 7.). The elevation of the necroptosis marker, RIPK1 [32] assessed the involvement of necroptosis. Since significant PARP cleavage could not be detected the involvement of apoptosis was not likely neither in ascorbate treatment nor in ferroptosis induction. These results further confirmed that both autophagy and necroptosis, but not apoptosis are involved in pharmacological ascorbate induced cell death.

Since pharmacologic ascorbate induced cytotoxicity and ferroptosis are both similarly characterised as an iron-dependent oxidative cell death we hypothesised whether there is any interference between the two mechanisms. This hypothesis was supported by the earlier observation that sorafenib a

multi kinase inhibitor and also a ferroptosis inductor [24, 23] and pharmacologic ascorbate showed synergistic anti-tumour effect against hepatocellular carcinoma [3]. Therefore, the effect of co-treatment of the RAS-mutant HT-1080 cell line with ascorbate and the ferroptosis activators RSL3 and erastin was investigated. Because of these previous findings it was a real surprise that at lower concentrations (0.1–0.5 mM) ascorbate behaved as an inhibitor of both RSL3 and erastin induced ferroptosis (Fig. 8). Although at the first time the anti-ferroptotic effect of ascorbate seemed surprising, it is not without any precedent. Similar anti-ferroptotic effect of ascorbate (at similar concentration) was described in acetaminophen induced liver cell death (ferroptosis) [25]. At higher ascorbate concentrations, the cytotoxic effect of ascorbate exceeded its protective effect (Fig. 8a). This cytotoxic effect was countered when pyruvate was also present in the medium consequently ascorbate concentration could be further increased in order to further protect cells from ferroptosis (Fig. 8a-b). It was reported that an ascorbate derivative 1'-methyl-ascorbigen could also mitigate the programmed cell death in lymphocytes [33]. Summarily ascorbate at low concentrations inhibited the effects of typical ferroptosis inducers and at pharmacologic concentration did not show any synergistic effect with them. Therefore it is worth to count with this protective effect of low concentration ascorbate on the ferroptosis inducers treated tumour cells.

The nature of the cytotoxicity induced by pharmacologic ascorbate is oxidative, involving reactive oxygen species (ROS) production and lipid peroxidation (LOOX) [19]. Ferroptosis induced by its specific activators (e.g. erastin, RSL3) is accompanied by these hallmarks as well [18, 19]. Therefore, the production of ROS in the form of hydroxyl radical ($HO^{\cdot}$) and the formation of lipid peroxides (LOOX) as a result of treatment with pharmacologic ascorbate and the ferroptosis inducer RSL3 was also investigated. The inhibitory effect of ascorbate on these hallmarks of ferroptosis was also studied. Our results show that both pharmacologic ascorbate and RSL3 induce ROS and lipid peroxide formation (Fig. 9). The co-treatment of RSL3 treated cells with low dose (0.1 mM) ascorbate significantly decreased the level of both ROS and lipid peroxide formation (Fig. 9). The production of ROS (Fig. 9a) LOOX (Fig. 9b) and the elevation of labile iron pool (LIP) (Fig. 9c) due to pharmacologic ascorbate treatment was concentration dependent. Ascorbate concentration as low as 0.25 mM induced detectable levels of ROS while cells treated with 0.1 mM ascorbate showed ROS levels similar to untreated cells (Fig. 9d).

Our results corroborate the previous findings that the cell death mechanisms induced by both pharmacologic ascorbate and RSL3 are of oxidative nature and accompanies by lipid peroxide formation however they induce cancer cell death by different mechanisms which do not show any synergism.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare no conflict of interest.

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